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Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin

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Abstract

Hematopoietic stem cell transplantation (HSCT) offers curative therapy for patients with hemoglobinopathies, congenital immunodeficiencies, and other conditions, possibly including AIDS. Autologous HSCT using genetically corrected cells would avoid the risk of graft-versus-host disease (GVHD), but the genotoxicity of conditioning remains a substantial barrier to the development of this approach. Here we report an internalizing immunotoxin targeting the hematopoietic-cell-restricted CD45 receptor that effectively conditions immunocompetent mice. A

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

R.P. conceived the study, designed and conducted the experiments, analyzed the data, and wrote the manuscript; B.S., J.H., A.S., D.B.S., Y.K., A.C., T.A.T., F.R., and M.K.M. conducted the experiments, analyzed the data, and reviewed the manuscript; D.J.R. and G.L.V. designed experiments and reviewed the manuscript; D.T.S. conceived the study, designed the experiments, analyzed the data, and wrote the manuscript.

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single dose of the immunotoxin, CD45–saporin (SAP), enabled efficient (>90%) engraftment of donor cells and full correction of a sickle-cell anemia model. In contrast to irradiation, CD45–SAP completely avoided neutropenia and anemia, spared bone marrow and thymic niches, enabling rapid recovery of T and B cells, preserved anti-fungal immunity, and had minimal overall toxicity. This non-genotoxic conditioning method may provide an attractive alternative to current conditioning regimens for HSCT in the treatment of non-malignant blood diseases.

HSCT, one of the fastest-growing hospital procedures in the United States¹, is widely used to treat hematological malignancies. However, the ability of HSCT to cure a broad range of non-malignant diseases is severely underutilized. Hemoglobinopathies, such as sickle cell anemia and thalassemia, which affect millions of patients globally, are curable by HSCT when stable mixed chimerism (>25% donor-derived leukocytes in peripheral blood) restores hemoglobin and red blood cell parameters to >95% of normal²; disease-free survival in such cases is >90%^{3–6}. In addition to hemoglobinopathies, the hematologic manifestations of other non-malignant conditions, such as Fanconi anemia⁷ and Wiskott-Aldrich syndrome⁸; genetic conditions that cause neurologic decline, such as metachromatic leukodystrophy⁹; and immunodeficiencies, such as adenosine deaminase severe combined immunodeficiency (SCID)¹⁰, can be cured by HSCT. Furthermore, HSCT may provide benefit in the treatment of type I diabetes¹¹ and AIDS¹² and for induction of immune tolerance in organ transplantation¹³. The obstacles to using allogeneic HSCT in these diverse conditions relate primarily to the frequency of life-threatening GVHD, of acute complications that result from the cytotoxic effects of conditioning, such as mucositis and infections, and of long-term, irreversible complications that arise from the genotoxic effects of conditioning.

Advances in gene therapy and genome editing are enabling new approaches to HSCT using a patient's own cells that have been genetically corrected *ex vivo*. Clinical evidence of benefit from this approach for immunodeficiencies, such as SCID (X-linked and adenosine deaminase)^{14,15}, chronic granulomatous disease^{16,17}, Wiskott-Aldrich syndrome¹⁸, metachromatic leukodystrophy⁹, autoimmune systemic sclerosis¹⁹, and hemoglobinopathies^{20,21} is encouraging. Autologous HSCT eliminates the risk of GVHD but not the toxicities of conditioning. For example, a gene therapy trial for hemoglobinopathy was complicated by grade 3 or greater toxicities in all six patients, including premature menopause in a teenage girl (as presented)²¹. Another recent trial of autologous HSCT, for systemic sclerosis, showed a survival benefit in some patients but also 10% immediate after-transplant mortality¹⁹. The purpose of conditioning is to deplete host hematopoietic stem cells (HSCs) in the bone marrow niche so that transplanted HSCs will engraft efficiently. Current conditioning regimens involve total body irradiation (TBI) and/or cytotoxic drugs, which are non-targeted and genotoxic and have multiple short- and long-term adverse effects^{22,23}. The toxicities of conditioning lessen the willingness of patients and healthcare providers to consider this therapy except under extreme circumstances. Therefore, advancing autologous HSCT to the many patients who might benefit will require solving the problem of pre-transplant conditioning.

A promising avenue for improving the safety of conditioning is the use of drugs, such as antibodies, that are specifically targeted to HSCs and other hematopoietic cells in the bone

marrow niche and that spare non-hematopoietic cells. Thus far, studies of targeted conditioning approaches in mice, a useful model organism for HSCT research, have shown either poor donor-cell engraftment or efficacy limited to immunocompromised backgrounds^{24–27}. A conditioning regimen that minimized off-target toxicity and immunosuppression while enabling efficient engraftment would greatly expand the curative application of HSCT.

Here we describe CD45–SAP, a hematopoietic-cell-specific immunotoxin consisting of saporin (SAP) conjugated to a CD45-targeting antibody. SAP is a catalytic *N*-glycosidase ribosome-inactivating protein that halts protein synthesis²⁸. Unlike other ricin family members, it lacks a general cell entry domain and is non-toxic unless conjugated to a targeting antibody or ligand capable of receptor-mediated internalization²⁸. SAP and other protein-based immunotoxins have been widely explored in cancer therapy, with greater success for hematological malignancies than for solid tumors^{29,30}. However, they have not been studied as a conditioning strategy in HSCT to our knowledge. We identified CD45–SAP from an *in vivo* HSC depletion screen exploring SAP-based immunotoxins targeted to various cell surface receptors present on HSCs. We show that CD45–SAP is an internalizing immunotoxin that efficiently conditions immunocompetent mice for autologous HSCT, minimizes undesirable toxicity and promotes rapid immunological recovery compared with conventional TBI conditioning.

RESULTS

CD45–SAP is a potent immunotoxin capable of depleting HSCs

To evaluate immunotoxins as a means of depleting endogenous HSCs from their niches, we targeted a set of cell-surface antigens present on mouse and human HSCs with SAP-based immunotoxins. We conducted our experiments in fully immunocompetent C57Bl/6 mice, a background that has proven challenging for antibody-based conditioning²⁶. Immunotoxins were prepared by combining appropriate biotinylated monoclonal antibodies with a streptavidin–SAP conjugate. To assess HSC depletion, we harvested bone marrow 8 d after intravenous injection of 3 mg/kg immunotoxin and quantified HSCs (Lin[–]cKit⁺Sca1⁺CD48[–]CD150⁺) by flow cytometry. (Fig. 1a). We evaluated seven candidate antigen targets known to be present on both murine and human HSCs in our *in vivo* screen: CD45, CD49d, CD84, CD90, CD133, CD135, and CD184. CD45–SAP was the most efficient in depleting bone marrow HSCs (Supplementary Fig. 1a).

Ratio and dose optimization studies (Fig. 1b and Supplementary Fig. 1b) identified a single CD45–SAP dose (by intravenous (i.v.) injection) of 3 mg/kg of 1:1 antibody to streptavidin–SAP ratio as achieving the highest immunophenotypic HSC depletion (98% by flow cytometry). The colony-forming activity of bone marrow progenitors decreased in a dose-dependent manner but was less adversely affected than HSCs (Fig. 1b). Competitive bone marrow transplantation confirmed the depletion of functional HSCs by CD45–SAP (Supplementary Fig. 1c). As expected, non-biotinylated CD45 antibody plus streptavidin–SAP did not deplete HSCs *in vivo* (Fig. 1c). Furthermore, as the CD45 monoclonal antibody employed (clone 104) selectively recognizes the CD45.2 isoform of mouse CD45, the immunotoxin was unable to deplete HSCs in CD45.1 congenic mice (Supplementary Fig.

1d). Together, these results are consistent with antigen-specific depletion of HSCs by CD45–SAP.

To further characterize CD45–SAP, we performed a series of *in vitro* cell death and internalization experiments using the mouse hematopoietic cell lines EML (a multi-potent hematopoietic progenitor line) and EL4 (a T-cell lymphoma line). EML cells resemble hematopoietic stem and progenitor cells as they are dependent on stem cell factor for growth and undergo multi-lineage differentiation upon cytokine stimulation. In addition to the CD45 antibody clone 104, we investigated clone 30-F11. Immunotoxins created from both clones potently induced EML and EL4 cell death with similar IC₅₀ (50% cell death) values ranging between 40 and 71 pM (Fig. 1d and Supplementary Fig. 1e). Control non-biotinylated antibody in the presence of streptavidin–SAP did not induce cell death *in vitro* (Fig. 1d), demonstrating that association of the SAP and the targeting antibody was required. Quantification of anti-CD45 antibody internalization in EL4 cells using clone 104 showed 7% internalization of the naked antibody and 12% internalization of antibody–streptavidin complex over a 24 h period (Fig. 1e). Surprisingly, despite equivalent activity of immunotoxins created from both anti-CD45 clones *in vitro*, only immunotoxin created from clone 104 was capable of efficient immunophenotypic HSC depletion *in vivo* (Supplementary Fig. 1f). Assessment of the *in vivo* persistence of the antibodies (24 h after administration) revealed that clone 104 prominently bound to peripheral white blood cells, splenocytes, and HSC-containing bone marrow Lin[−]cKit⁺Sca1⁺ (LKS) cells, whereas clone 30-F11 displayed poor persistence *in vivo* (Supplementary Fig. 1g).

Taken together, these results suggest that *in vivo* binding and internalization of CD45–SAP efficiently depletes HSCs from the mouse bone marrow.

CD45–SAP enables efficient donor-cell engraftment

We next determined whether HSC depletion by CD45–SAP enables engraftment of donor cells. As the donor graft may be negatively affected by unbound CD45–SAP *in vivo*, we varied the time of transplantation to identify the optimal transplantation window (Fig. 2a) and explored transplantation of two donor cell types (in different cohorts): congenic CD45.1 cells, which cannot be targeted by CD45–SAP, and syngeneic CD45.2–GFP cells, which can potentially be targeted. A dose of ten million bone marrow donor cells was used for transplantation, consistent with prior mouse reduced-intensity conditioning studies^{24,31,32}. This dose corresponds to ~2% of total mouse marrow³³, thereby resembling human transplantation in which ~5% of donor marrow is harvested³⁴.

Four months after transplantation, we observed 75–90% donor cell (GFP⁺ or CD45.1⁺ cells) chimerism within the total white blood cells of the peripheral blood as assessed by flow cytometry for both donor cell types in mice conditioned with 3 mg/kg CD45–SAP (Fig. 2b,c). Notably, equivalent levels of chimerism were observed for cells transplanted between 2 and 12 d after CD45–SAP administration (Fig. 2b), demonstrating a wide transplantation window. Unconditioned control mice did not demonstrate meaningful donor-cell engraftment, with chimerism levels <2% (Fig. 2b,c). Similar to peripheral blood chimerism, chimerism in bone marrow HSCs in CD45–SAP-conditioned mice at 4 months after transplantation was 90% (Supplementary Fig. 2a). Time-course assessment revealed that

peripheral chimerism was stable and reached 93–94% at 15 months for both donor cell types (Fig. 2d and Supplementary Fig. 2b). Myeloid chimerism was established rapidly, within 2 months, and as expected the highest levels of T- and B-cell donor chimerism appeared after longer intervals, likely due to the longer lifespan of resident host lymphoid cells (Supplementary Fig. 2c). Analysis of peripheral blood at 8 months after transplantation revealed a normal distribution of the myeloid, B- and T-cell lineages, indicative of truly non-biased stem cell engraftment (Fig. 2e). Stem cell engraftment was further confirmed by serial transplantation into lethally irradiated secondary recipients (Supplementary Fig. 2d). Notably, conditioning with CD45–SAP was not mouse-strain-specific as both C57BL/6 and BALB/c recipients had high levels of donor-cell engraftment (Supplementary Fig. 2e,f). HSCs may be immunophenotypically defined as LKS CD34⁺CD150⁺, or, alternatively, as LKS CD48⁺CD150⁺. In addition to bone marrow cell transplants, we tested whether CD45–SAP conditioning also enabled engraftment of purified HSCs. Transplantation of 2,000 LKS CD34⁺CD150⁺ HSCs or LKS CD48⁺CD150⁺ HSCs yielded 60% myeloid chimerism (Fig. 2f and Supplementary Fig. 2g) at 4 months, whereas non-conditioned control mice transplanted with HSCs did not demonstrate meaningful engraftment (0–0.03% chimerism).

To compare CD45–SAP with other conditioning methods, we investigated conventional TBI and experimental antibody-based conditioning using the ACK2 monoclonal antibody, a CD117 antagonist²⁵. The chimerism achieved 4 months after transplantation by 3 mg/kg CD45–SAP in wild-type mice matched that of 5Gy TBI (50% of lethal TBI dose) conditioning (Supplementary Fig. 2h) and was multi-lineage (Supplementary Fig. 2i). Conditioning with 28 mg/kg ACK2 did not enable substantial engraftment (<3% engraftment) in this immunocompetent background (Supplementary Fig. 2h), consistent with previous observations²⁶. Moreover, immunotoxin created from ACK2 (ACK2–SAP; 3 mg/kg) was also inactive (Supplementary Fig. 2h). Injection of one-tenth the cell dose (one million bone marrow cells) confirmed that 3 mg/kg CD45–SAP and 5Gy TBI achieve equivalent engraftment (≈20% chimerism) at this lower cell dose, with significant synergy (≈90% chimerism) when the conditioning methods were combined (Supplementary Fig. 2j).

Assessment of peripheral myeloid, B-, and T-cell recovery after transplantation demonstrated that CD45–SAP conditioning enabled quicker recovery of these cell types versus irradiation-based conditioning (Supplementary Fig. 3), and the graft was found to contribute to various T-cell subpopulations (Supplementary Fig. 4) in CD45–SAP-conditioned mice. All donor-derived T-cell subsets examined were significantly elevated compared with the same subsets in non-conditioned control mice ($P < 0.001$ in all subsets).

CD45–SAP preserves the normal bone marrow architecture

As 3 mg/kg CD45–SAP and 5Gy TBI yielded equivalent levels of chimerism, we determined the toxicities of these two conditioning approaches by measuring various blood and bone marrow parameters in conditioned mice that did not undergo after-conditioning transplantation. Both CD45–SAP and 5Gy TBI were not fully myeloablative as they permitted long-term survival (>6 months) without stem cell transplantation ($n = 12$ mice/group, data not shown). Time course assessment after conditioning revealed that CD45–SAP had significantly ($P < 0.01$) less adverse immediate effects on bone marrow cellularity (Fig.

3a), with quicker recovery to normal levels than irradiation (6 vs. 12 d for CD45–SAP vs. irradiation, respectively). Similarly, the effect of CD45–SAP on bone marrow progenitor cells was less profound than that of irradiation as measured by *in vitro* colony-forming cell activity assays (Fig. 3b). Assessment of specific progenitor types in the bone marrow revealed differential effects following 3 mg/kg CD45–SAP versus 5Gy TBI (Supplementary Fig. 5). Notably, myeloid progenitors, granulocyte macrophage progenitors, common myeloid progenitors, and common lymphoid progenitors were less adversely affected or recovered more quickly after CD45–SAP. Despite the overall reduced toxicity of CD45–SAP toward bone marrow cellularity and short-term progenitors, CD45–SAP depleted HSCs as efficiently as irradiation (~98% immunophenotypic depletion, Fig. 3c), although not as rapidly.

Femur histology performed 2 d after conditioning suggested that CD45–SAP outperforms irradiation not only in preserving bone marrow cellularity but also in maintaining vascular integrity within the marrow, as red blood cells remained within blood vessels, similar to observations in non-treated control mice (Supplementary Fig. 6). In contrast, 5Gy-irradiated mice had lower levels of nucleated cells within the marrow, with dispersion of red blood cells throughout, indicating gross disruption of the vasculature. To confirm these differences, we performed a functional assay to assess vascular integrity. High molecular weight (2 MDa) rhodamine–dextran was injected intravenously 2 d after conditioning, and intravital imaging of the calvarium bone marrow was performed. Rhodamine–dextran was effectively retained within the blood vessels of mice conditioned with CD45–SAP, similar to unconditioned control mice, suggesting maintenance of vascular integrity (Fig. 3d). Irradiated recipients, however, exhibited diffuse rhodamine–dextran throughout the marrow, indicative of compromised vascular integrity. The non-hematopoietic composition of the marrow (measured as the ratio of endothelial cells to stromal cells) was significantly ($P < 0.001$) altered in irradiated recipients, whereas the composition in immunotoxin-treated recipients was similar to that of non-treated control mice (Supplementary Fig. 7). Assessment of endosteal cells 2 d after conditioning revealed similarities between CD45–SAP treatment and non-treated control mice, with preservation of endosteal cell morphology (Supplementary Fig. 8). In irradiated recipients, altered morphology of endosteal cells was observed, as has been documented by others³⁵ (Supplementary Fig. 8).

Together, these results indicate that CD45–SAP was less detrimental to bone marrow cellularity, hematopoietic progenitors, and the marrow microenvironment than 5Gy irradiation, while achieving efficient HSC depletion and allowing similar levels of engraftment.

CD45–SAP permits more rapid immune recovery

Recovery of adaptive and innate immunity is of paramount importance for survival after HSCT, and its failure after conditioning contributes to the morbidity and mortality associated with transplantation. Analysis of the peripheral blood following conditioning (without transplantation) revealed significant ($P < 0.05$) differences between 3 mg/kg CD45–SAP and 5Gy TBI conditioning. Whereas irradiation suppressed myeloid (Mac1⁺, Gr1⁺) cells for 28 d, CD45–SAP-conditioned mice showed an immediate and sizable increase (threefold) in

circulating myeloid cells that returned to normal levels at 12 d (Fig. 4a). To test innate immunity, we challenged non-transplanted conditioned mice with a systemic infection of *Candida albicans* (2 d after conditioning), a clinically relevant fungal strain that infects immunocompromised HSCT patients after conditioning. Mice conditioned with 5Gy TBI were highly susceptible to candida challenge, with 100% lethality occurring within 3 d after infection, (Fig. 4b, P value vs. control <0.0001 log-rank Mantel-Cox test). In contrast, mice conditioned with 3 mg/kg CD45–SAP were considerably more resilient (P value vs. irradiation <0.0002 log-rank Mantel-Cox test) with overall survival over 50 d similar to that of naive non-conditioned control mice (P value of control vs. CD45–SAP = 0.57 log-rank Mantel-Cox test). Therefore, innate immune function was similar to that of control non-conditioned mice in protection against fungal infection.

Both B and T cells were equally and potently depleted 2 d after conditioning by 3 mg/kg CD45–SAP and 5Gy irradiation (Fig. 4c and Supplementary Fig. 9a). However, recovery of lymphocytes was considerably more rapid in non-transplanted CD45–SAP-treated mice. B cells recovered to 80% within 18 d (Supplementary Fig. 9a), and T cells recovered to 70% within 12 d (Fig. 4c). In contrast, non-transplanted irradiated mice required 48 d for B and T cells to recover to similar levels (Fig. 4c and Supplementary Fig. 9a). The faster T-cell recovery observed with CD45–SAP may be due to differential effects on the thymus, an organ critical for the generation of new T cells, which is typically damaged by TBI conditioning³⁶. Histology of thymi 2 d after conditioning in non-transplanted mice demonstrated that irradiation induced visible thymic atrophy with considerable reduction of thymocyte cellularity in the cortex, whereas no thymic atrophy was evident following CD45–SAP conditioning (Fig. 4d). We tested preservation of thymic function by measuring the presence of T-cell receptor excision circles (TRECs), the molecular signature of T-cell receptor rearrangement that does not replicate with genomic DNA and therefore marks newly generated T cells. Quantification of TRECs per mg of thymus tissue 3 d after conditioning in non-transplanted mice (Fig. 4e) revealed *de novo* T-cell output following CD45–SAP treatment was 84% of normal (P value vs. control = 0.025), whereas 5Gy TBI decreased T-cell output to 8% (P value vs. control <0.0001). Irradiation also reduced thymic mass by 80%, whereas CD45–SAP decreased thymic mass by 50% (Supplementary Fig. 9b). These data are consistent with relative preservation of T-cell neogenesis after CD45–SAP treatment and moderately depleted thymic cellularity.

CD45–SAP (3 mg/kg) did not induce anemia, as red blood cell, hematocrit, and hemoglobin levels remained normal, whereas irradiation induced mild anemia 6 d after conditioning (Supplementary Fig. 9c–e). Platelets were mildly affected by both conditioning regimens, with a decrease to 40% of normal levels (Supplementary Fig. 9f). No observable toxicity was observed in the gastrointestinal tract, liver, and ovaries for either CD45–SAP or irradiation as assessed by necropsy and histology (data not shown).

Taken together, our results suggest that CD45–SAP conditioning preserves myeloid innate immunity, avoids anemia, and facilitates rapid B- and T-lymphocyte recovery versus the equivalent conditioning dose of irradiation.

CD45–SAP enables correction of sickle cell disease

To investigate whether CD45–SAP conditioning could enable curative transplantation in a clinically relevant non-malignant hemoglobinopathy model, we used knock-in mice bearing the human sickle hemoglobin gene, which mimic human sickle cell disease. These mice exhibit decreased red blood cell counts, hematocrit, and hemoglobin levels, with elevated numbers of immature red blood cells (reticulocytes) and abnormally large spleens³⁷. Previous transplantation studies in sickle mice have shown that 25% myeloid chimerism returns blood parameters to 90% of normal and that 70% myeloid chimerism is needed to completely correct organ pathophysiology³⁸.

As sickle mice have elevated white blood cell levels (versus wild-type mice), we re-optimized the dose of CD45–SAP and determined that a single dose of 4 mg/kg at day 0 or two sequential doses of 3 mg/kg of CD45–SAP on days 0 and 3 achieved maximal stem cell depletion ($\approx 99\%$ immunophenotypic depletion at day 8, Supplementary Fig. 10a) in the bone marrow. Using these doses, we investigated three transplantation protocols (groups A–C; six mice/group) (Fig. 5a). All mice in the three groups (18/18 mice) conditioned with CD45–SAP and transplanted with wild-type cells demonstrated $>90\%$ donor myeloid chimerism at 4 months after transplantation (Fig. 5b). A complete normalization of red blood cell, hemoglobin, hematocrit, and reticulocyte levels was also achieved after transplantation (Fig. 5c and Supplementary Fig. 10b–e).

Sickle hemoglobin protein in the blood was completely replaced with normal hemoglobin protein as assessed by native-PAGE analysis (Fig. 5d and Supplementary Fig. 10f). In addition, blood smears showed a lack of sickle-shaped red blood cells, unlike blood smears of sickle control mice (Fig. 5e), and spleen sizes returned to normal (Fig. 5f and Supplementary Fig. 10g). Therefore, CD45–SAP conditioning achieves $>90\%$ myeloid chimerism with full disease correction of sickle cell anemia after transplantation.

DISCUSSION

Current conditioning regimens for HSCT involve varying doses of chemotherapy, either alone or in combination with irradiation. These cytotoxic and genotoxic interventions have minimal cell selectivity and cause extensive collateral damage to healthy tissue. Thrombocytopenia and anemia can be readily treated with platelet or red blood cell transfusions^{9,18}, but neutropenia and lymphopenia cannot, leaving patients susceptible to severe infection^{39,40}. Of greater concern is genetic injury to non-hematologic and hematologic tissues. Acute genetic toxicities to the skin, gut, and airway manifest as hair loss, diarrhea, mucositis, and disrupted barrier to infection^{22,23}. Long-term risks include such consequences as infertility, stunted skeletal and brain development, and cancer.

Emerging antibody-based conditioning agents are expected to have much less off-target toxicity than traditional, non-targeted modes of cell killing. In the best-case scenario, HSC-specific immunotoxins would selectively deplete HSCs and preserve adaptive and innate immunities while avoiding thrombocytopenia, anemia, and damage to hematopoietic microenvironments. Previous studies have supported the promise of antibody-based conditioning. For example, ACK2, an anti-CD117 naked antagonist antibody, enabled

autologous transplantation in immunocompromised mice²⁵. However, high-dose ACK2 failed to condition immunocompetent adult mice and induced anemia, neutropenia, and thrombocytopenia⁴¹. Although some efficacy was observed in fetal mice⁴², ACK2 required combination with sub-lethal irradiation to achieve meaningful chimerism in adult immunocompetent mice²⁶ and this combination showed modest efficacy in non-human primates⁴³. In addition, CD117 is expressed on cardiac progenitors, gastrointestinal cells, neuronal cells, and cells of the reproductive system, raising concerns about potential off-target toxicity⁴⁴.

We chose the antibody target CD45 on the basis of our results from an *in vivo* screen of seven hematopoietic markers. CD45 is expressed exclusively by all hematopoietic cells with the exception of platelets and erythrocytes⁴⁵. Anti-CD45 radioimmunotherapy is currently under clinical investigation as a myeloablative alternative to conventional conditioning in patients with acute myeloid leukemia and myelodysplastic syndrome. However, a major concern with this agent is bystander toxicity, whereby non-target cells, particularly those in the bone marrow and thymus, are affected by irradiation owing to their close proximity to target cells. Similar to conventional conditioning, anti-CD45 radioimmunotherapy induces neutropenia, lymphopenia, and thrombocytopenia^{46,47}, and damage to the bone marrow microenvironment is highly likely. Naked antibodies targeting CD45 have also been tested for conditioning in HSCT. However, they depleted only lymphoid cells (perhaps through complement-dependent cytotoxicity), and additional DNA-targeting chemotherapy was required to deplete HSCs^{48,49}; such combinations have been tested in human transplantation but required genotoxins for efficacy⁵⁰.

In contrast, our study demonstrates that single-entity targeting of CD45 using a protein-based immunotoxin achieves HSC depletion in fully immunocompetent mice and enables transplantation while avoiding neutropenia, anemia, and thymopoietic damage. The requirement for receptor-mediated internalization of CD45–SAP to achieve cell killing limits the risks of off-target and bystander toxicity. CD45–SAP promoted rapid marrow and peripheral lymphocyte regeneration, at least in part by avoiding toxicity to non-target niche cells. We successfully used CD45–SAP to condition sickle disease mice and demonstrated full disease correction after transplantation. These results suggest that protein-based immunotoxins may be preferred for non-malignant conditions in which stable mixed chimerism is sufficient to cure the underlying disease (e.g., hemoglobinopathies and SCID conditions). Additionally, the enhanced stability and cost-effective production of protein-based immunotoxins compared to radioimmunotherapy may facilitate their widespread use. Finally, as protein-based immunotoxins such as CD45–SAP do not induce DNA damage, they may be better suited to condition pre-malignant Fanconi anemia patients, who are genetically predisposed to be hypersensitive to DNA damaging agents and conventional conditioning⁵¹.

The impact of CD45–SAP on HSCs was markedly different than on progenitors, as demonstrated by flow cytometry and colony-forming capacity (Fig. 3 and Supplementary Fig. 5). However, expression of the CD45 antigen on the surface of HSCs and various progenitor populations did not closely correlate with relative sensitivity to CD45–SAP in our hands (Supplementary Fig. 11), a result that may be explained by cell-specific differential

processing of antibody-engaged CD45. Future studies will explore the dependence of internalization kinetics and subsequent processing on cell differentiation state.

The experiments described in this study are relevant to autologous HSCT, and further experimentation is required to determine the utility of CD45 immunotoxins in allogeneic transplantation. It is conceivable that other targets, including those investigated in our initial screen, may enable efficient conditioning upon further optimization and that our approach may lead to HSC-specific immunotoxins that completely preserve immunity while enabling autologous HSCT. Toward this goal, this study offers several considerations. As HSCs are predominantly in a quiescent non-dividing state⁵², it will be of interest to determine whether toxins that inhibit protein synthesis (e.g., SAP, modified ricin analogs, pseudomonas, or diphtheria toxin) are particularly effective for HSC depletion as they induce death independent of the cell cycle⁵³, in contrast to anti-mitotic small molecules currently used in antibody-drug conjugates⁵⁴. Although a previous study suggested that CD45 would be an unsuitable target for internalizing immunotoxins due to its poor internalization frequency⁵⁵, we observed that 12% internalization (measured over a 24 h period *in vitro*, Fig. 1e) is sufficient to achieve potent HSC depletion with *in vivo* utility. As CD45 is highly expressed at 200,000 molecules per leukocyte⁵⁶, the absolute number of internalized molecules, rather than internalization frequency alone, likely determines target suitability. Furthermore, in addition to favorable *in vivo* persistence, high-affinity immunotoxins that minimize shedding and undesirable targeting of donor cells may be required to achieve a wide transplantation window.

In cancer therapy, the clinical utility of immunotoxins has been limited by issues of immunogenicity and cumulative dose-limiting toxicity⁵⁷. These factors may be less relevant to pre-HSCT conditioning, which will likely require limited administration of immunotoxin. Furthermore, the wealth of safety data available from previous immunotoxin clinical trials for hematological malignancies may facilitate clinical translation of immunotoxins for conditioning. Taken together, our results indicate that CD45–SAP or similar protein-based immunotoxins may benefit patients undergoing autologous HSCT by minimizing the toxicities of conditioning regimens.

To deliver on the promise of this approach to patient care, it obviously must be effective against human cells. We have begun testing antibodies against human CD45 on human hematopoietic cell lines. Two such antibodies were capable of potently inducing cell death with IC₅₀ values of 130 and 200 pM (Supplementary Fig. 12), demonstrating that anti-CD45 antibody internalization is not species-specific.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

General methods and statistics

Sample sizes for animal studies (typically $n = 5$ mice/group within each experiment) were based on prior similar work without the use of additional statistical estimations. All statistics were calculated using unpaired Student *t*-test using two-sided analysis except Kaplan-Meier data, which were analyzed by log-rank (Mantel-Cox) test. Alphanumeric coding was used to blind pathology samples and CFC counting.

Animal studies

All animal studies were performed with Harvard faculty of arts and sciences (FAS) or Massachusetts General Hospital IACUC approval. Wild-type CD45.2 mice (C57BL/6J and BALB/cByJ), congenic CD45.1 mice (B6.SJL-Ptprca Pepcb/BoyJ and CByJ.SJL(B6)-Ptprca/J), GFP mice (CByJ. B6-Tg(UBC-GFP)30Scha/J), and sickle mice (B6;129-Hb^{tm1}(HBA)Tow Hb^{tm2}(HBG1, HBB*)Tow/Hb^{tm3}(HBG1, HBB)Tow/J) were purchased from Jackson Laboratories. Female mice were used for all experiments at approximately 7–9 weeks of age, unless stated otherwise. All mice within each experiment were age-matched and no randomization was performed. Pre-established criterion for animal omission was failure to inject >90% of desired immunotoxin/cell dose. Health concerns unrelated to the procedure (e.g., malocclusion, severe dermatitis) were criteria for omission and euthanasia. For sickle experiments, sickle chimeras were created by transplanting lethally irradiated (9.5Gy single dose, cesium-137 irradiator, JL Shephard & Associates) 6-week-old C57BL/6J mice with 5 million bone marrow (BM) cells harvested from sickle mice. Immunotoxin conditioning and transplantation in sickle chimeras were performed 8 weeks after chimera creation.

Antibodies and immunotoxin preparation

Biotinylated anti-CD45 (clones 30-F11 and HI30)^{49,58}, anti-CD45.2 (clone 104)⁵⁹, anti-CD49d (clones 9C10)⁶⁰, and anti-CD84 (clone mCD84.7)⁶¹ monoclonal antibodies were purchased from BioLegend. Biotinylated anti-CD90 (clone 30-H12)⁶², anti-CD133 (clone 13A4)⁶³, anti-CD184 (clone 2B11)⁶⁴, and anti-CD135 (clone A2F10)⁶⁵ monoclonal antibodies were purchased from eBioscience. Biotinylated anti-CD45 (clone MEM-28)⁶⁶ monoclonal antibody was purchased from Novus Biologicals. Anti-CD117 antagonist antibody (clone ACK2)²⁵ was purchased from BioLegend and injected at 28 mg/kg. Immunotoxins were prepared by combining biotinylated antibodies (160 kDa MW) with streptavidin–SAP conjugate (2.8 saporin molecules per streptavidin, 135 kDa MW, Advanced Targeting Systems) in a 1:1 molar ratio and subsequently diluted in PBS immediately before use. Dose calculations assumed a combined molecular weight of 295 kDa for the immunotoxins. *In vivo* administration of immunotoxin was performed by intravenous injections (300 μ L volume).

In vitro cell death assay

In vitro cell death experiments were performed using EL4 (ATCC TIB-39), EML (ATCC CRL-11691) and Jurkat (clone E6-1, ATCC TIB-152) cell lines. The morphology, growth

kinetics, and immunophenotype (e.g., EL4 and EML by mouse CD45 expression; EML by murine stem cell factor-dependent growth; and Jurkat by human CD45 and CD3 expression) of the cell lines were consistent with established parameters and mycoplasma testing was not performed. Jurkat cells were confirmed to express human CD45. Cells were plated in 96-well plates with 5,000 cells/well in 100 μ L volume cell culture media containing various concentrations of immunotoxin. Three independent experiments were performed with three technical replicates within each experiment. After 72 h, cell viability was determined using the CellTiter MTS assay (Promega). PBS-treated and 10 μ M staurosporine-treated cells (Sigma-Aldrich) were used as live and dead controls, respectively.

Measurement of antibody internalization

In vitro antibody internalization was assessed as previously described⁶⁷. Briefly, EL4 cells (200,000/mL) in complete media (RPMI w/o phenol red, 10% FBS) were plated into 96-well plates with 20 nM AF488-labeled anti-CD45.2 antibody (clone 104, BioLegend) or a 1:1 mixture of biotinylated anti-CD45.2 antibody and streptavidin-AF488 conjugate (Life Technologies) with $n = 6$ technical replicates. After 24 h of incubation, the cells were washed twice and resuspended in PBS containing 2% FBS. Samples were split into two and one-half was incubated with 0.25 mg/mL polyclonal anti-AF488 quenching antibody (clone A-11094, Life Technologies). AF488 signal in samples with and without quenching antibody was quantitated by flow cytometry. Unstained and time zero controls were performed to determine the quenching efficiency and calculate internalization frequency.

In vivo antibody persistence

C57BL/6 mice were i.v. injected with streptavidin-AF488 conjugate premixed with biotinylated anti-CD45 antibody (1:1 ratio in PBS, 1.8 mg/kg). Twenty-four hours after administration, blood, BM, and spleen were harvested and AF488 signal determined by flow cytometry. BM cells were stained with lineage cocktail (BD Biosciences), anti-cKit and anti-Sca1 antibodies in order to determine AF488 signal within the Lin⁻cKit⁺Sca1⁺ (LKS) progenitor population. AF488 signal in splenocytes and peripheral blood cells was assessed within the CD45⁺ cell fraction following *ex vivo* staining with anti-CD45 PeCy7 antibody.

BM analysis and transplantation

BM cells for transplantation or analysis were harvested by crushing all limbs or one femur, respectively. Total cellularity was determined by complete blood cell counting (CBC) analysis, using an Abaxis VetScan HM5 instrument. Progenitor colony assays were performed following manufacturer's instructions (Stem Cell Technologies). Immunophenotypic cKit, anti-Sca1, anti-CD48, and anti-CD150 antibodies and stem cells were defined as Lin⁻cKit⁺Sca1⁺CD48⁻CD150⁺. For non-hematopoietic cell analysis, flushed bone marrow plugs from long bones were digested using 2 mg/ml dispase and 1 mg/ml collagenase IV enzymatic cocktail in Hanks' balanced salt solution (HBSS) for 2 $\times$ 15 min cycle at 37 $^{\circ}$ C. Flushed bones were then crushed, cut into small pieces and digested with 0.25% collagenase (1 h 37 $^{\circ}$ C) with shaking. Endothelial and stromal populations in the marrow and collagenase fractions were defined by flow cytometry using CD45-Ter119-CD31⁺ and CD45⁻Ter119-CD31⁻ gating, respectively. Anti-CD45 mAb (clone 30-F11) was used for cytometry analysis to avoid potential interference from CD45-SAP (created from

anti-CD45.2 mAb clone 104). For BM transplants, 10 million cells in 300 μ L PBS were injected intravenously. For purified stem cells injections, BM cells were lineage depleted by magnetic selection (BD Biosciences) before FACS sorting of LKS CD48⁻CD150⁺ or LKS CD34⁻CD150⁺ cells. Two thousand purified stem cells were injected per mouse. Secondary transplants were performed by injecting 1 million BM cells from primary conditioned and transplanted mice (4 months after transplantation) and injected into secondary lethally irradiated recipients (9.5Gy single dose). Competitive transplants were performed by injecting 1 million BM cells containing a 1:1 ratio of CD45.1 competitor and CD45.2 test cells into lethally irradiated (9.5Gy single dose) congenic CD45.1 recipients.

Peripheral blood analysis

Cohorts of mice (typically 4 mice/group) were serially bled or terminally bled by cardiac bleed. White blood cell, hemoglobin, red blood cell, platelet, and hematocrit levels were quantified by CBC analysis (Abaxis VetScan HM5). For flow cytometry quantification of T, B, and myeloid cells, blood samples were red blood cell-lysed and fixed before staining with anti-CD45, -B220, -CD3, -Mac1, and -Gr1 antibodies and absolute numbers of T, B, and myeloid cells were calculated using flow cytometry frequencies and white blood cell values obtained by CBC analysis. Peripheral blood donor chimerism was determined by flow cytometry using anti-CD45.1 and CD45.2 antibodies for transplants involving CD45.1 donor cells. For transplants using CD45.2-GFP donor cells, chimerism was based on GFP⁺ events within the CD45⁺ gate. Reticulocyte frequency within the red blood cell population was determined by flow cytometry using thiazole orange staining (Retic-COUNT reagent, BD Biosciences). Native-PAGE analysis of hemoglobin protein was performed on any-kD precast gels (Bio-Rad) using lysed whole blood samples.

Pathology and histology

At various time points (2, 4, 6, and 8 d after conditioning) C57BL/6 mice were euthanized, fixed in Bouin's solution (Sigma-Aldrich) and submitted for necropsy and histology. Two independent experiments were performed with 1 mouse/group. Hematoxylin and eosin staining was performed on paraffin embedded sections of the liver, spleen, femur, kidney, intestinal tract, lymph nodes, thymus, and ovaries for assessment of toxicity. Representative images shown are consistent between the two independent experiments.

Intravital imaging of vascular integrity

Live imaging of the mouse calvarial BM vasculature of conditioned C57BL/6 mice (2 d after conditioning) or non-treated control mice was performed using a custom made multi-photon microscope⁶⁸ (Thorlabs, Inc.) incorporating a high pulse energy fiber-based femtosecond laser (Cazadero FLCPA, Calmar laser) with excitation wavelengths set at 775 and 950 nm. A water-immersed 60 $\times$ /1.00w objective (LUMPLFLN60XW, Olympus) provided a 415 $\times$ 415 μ m field of view and 0.5–5 μ m Z-steps were used to a depth of 150–200 μ m. Mice were maintained under anesthesia (1.35% isoflurane/oxygen mixture) and body core temperature was maintained using a warmed plate. A U-shaped incision on the scalp exposed the calvarium bone, to which 2% methocellulose gel was applied for refractive index matching. Second harmonic generation signal (excited at 387.5 nm) was used to visualize bone collagen and to determine a region of interest. On-stage retro-orbital injection of 2 MDa

rhodamine-dextran conjugate (150 μ L of 3.3 mg/mL D-7139, Life Technologies) was performed and rhodamine signal (585 nm excitation) was continuously recorded (13 frames/second) for the first 2–5 min after injection. Serial images were collected up to 30 min after injection. Images taken at similar times after rhodamine-dextran administration were used for comparison between groups and two independent experiments were performed with 1 mouse/group/experiment. Contrast and brightness settings of the images in the figures were adjusted for display purposes only.

Systemic challenge with *Candida albicans*

Candida albicans, wild-type stain SC5314 (ATCC MYA-2876), was grown overnight from frozen stocks in yeast extract, peptone, and dextrose (YPD) medium (BD Biosciences) with 100 μ g/mL ampicillin (Sigma) in an orbital shaker at 30 °C. After pelleting and washing with cold PBS, yeast were counted using a hemocytometer and cell density adjusted in PBS to 75,000 CFUs per 200 μ L. C57BL/6 mice (non-conditioned control or 2 d post 3 mg/kg CD45–SAP or 5Gy TBI) were injected via lateral tail vein with 75,000 CFUs and animals were monitored daily. Moribund mice were euthanized humanely.

Quantification of thymic T-cell receptor excision circles (TRECs)

TREC quantification was performed as previously described⁶⁹. Briefly, thymi were harvested from non-conditioned C57BL/6 mice, 5Gy TBI or 3 mg/kg CD45–SAP conditioned mice (3 d after conditioning). Total DNA was extracted using TRIZOL following tissue homogenization in a Bullet Blender Storm BBX24 instrument (Next Advance, Inc.). DNA was quantified by UV-Vis and 1 μ g of DNA per sample was used as input for real-time PCR. A standard curve of mouse sjTREC plasmid was used to calculate the absolute number of single joint TRECS (sjTRECs) per sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CD45-SAP has potent cell-depletion activity. **(a)** Experimental outline for assessing ability of immunotoxins to deplete HSCs in immunocompetent C57BL/6 mice. HSCs were assessed by flow cytometry ($\text{Lin}^- \text{cKit}^+ \text{Sca1}^+ \text{CD48}^- \text{CD150}^+$) and progenitor colony forming cells (CFCs) were assessed by colony forming assay. **(b)** Dose-dependent effects of CD45-SAP on HSCs and CFCs, assessed 8 d after administration in C57BL/6 mice. Non-treated mice served as the control. Data represent mean $\pm$ s.d. ($n = 30$ mice, 5 mice/group, assayed individually); all data points significant vs. control ($P > 0.05$). **(c)** CD45-SAP depletes HSCs in C57BL/6 mice whereas non-biotinylated CD45 antibody in the presence of streptavidin-SAP does not. Data represent mean $\pm$ s.d. ($n = 5$ mice/group, one of two independent experiments shown). **(d)** CD45-SAP clone 104 kills EML progenitor cells *in vitro* (72 h incubation) whereas non-biotinylated antibody in the presence of streptavidin-SAP does not affect cell viability. Data represent mean $\pm$ s.d. ($n = 3$ technical replicates) of one of three independent experiments. **(e)** Percent internalization of clone 104 antibody (Ab alone) or antibody-streptavidin complex (Ab-streptavidin) in EL4 cells over 24 h *in vitro* culture. Data represent mean $\pm$ s.d. of a single experiment with $n = 6$ replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s., not significant ($P > 0.05$). Statistics calculated using unpaired *t* test.

Figure 2.

CD45-SAP enables efficient donor-cell engraftment. **(a)** Experimental outline for assessing transplantation window following 3 mg/kg CD45-SAP conditioning of C57BL/6 mice and transplantation of either CD45.1 or CD45.2-GFP 10^7 bone marrow cells. **(b)** Peripheral blood donor chimerism 4 months after transplantation of CD45.2 GFP or CD45.1 cells injected at various time points after 3 mg/kg CD45-SAP conditioning of C57BL/6 mice. Control represents non-conditioned mice receiving transplant. Data represent mean $\pm$ s.d. ($n = 30$ mice per donor cell type, 5 mice/time point, assayed individually); all data points significant vs. control ($P < 0.05$). **(c)** Representative flow cytometry plots illustrating donor cells in peripheral blood 8 months after transplantation in control or CD45-SAP conditioned C57BL/6 mice. **(d)** Long-term assessment of peripheral blood chimerism following CD45.2-GFP cell transplantation 8 d post CD45-SAP conditioning; all data points significant vs. control ($P < 0.05$). Data represent mean $\pm$ s.d. ($n = 5$ mice/group, assayed individually). **(e)** Contribution of donor cells to peripheral myeloid, B and T cells in CD45-SAP conditioned C57BL/6 mice 8 months after transplantation versus overall lineage distribution in non-treated control mice. Data represent mean $\pm$ s.d. ($n = 5$ mice/group, assayed individually) of one of two independent experiments. **(f)** Donor peripheral myeloid chimerism 4 months after transplantation of 2,000 purified HSCs (LKS CD48-CD150 $^{+}$ or LKS CD34-CD150 $^{+}$) in non-conditioned control and CD45-SAP conditioned C57BL/6 mice. Data represent mean $\pm$ s.e.m. ($n = 5$ mice/group, 2 independent experiments). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s., not significant ($P > 0.05$). Statistics calculated using unpaired t test.

Figure 3.

Differential effects of CD45-SAP and irradiation on bone marrow. **(a)** Relative bone marrow cellularity (total nucleated cells extracted from femur and tibia) at various time points after 3 mg/kg CD45-SAP or 5Gy TBI. **(b)** Relative CFC activity of bone marrow cells harvested from C57BL/6 mice at various times post 3 mg/kg CD45-SAP or 5Gy TBI. **(c)** Relative immunophenotypic quantification of HSCs in bone marrow harvested from C57BL/6 mice at various times post 3 mg/kg CD45-SAP or 5Gy TBI. Data in **a**, **b**, and **c** represent mean percentage relative to non-treated mice $\pm$ s.e.m. ($n = 12$ mice/group, $n = 4$ mice per time point, assayed individually). **(d)** Non-treated C57BL/6 control mice, or mice treated with 3 mg/kg CD45-SAP or 5Gy TBI (2 d after conditioning) were i.v. injected with high molecular weight (2 MDa) rhodamine-dextran to assess calvaria vascular integrity using intravital microscopy. Calvaria bone surface is shown in blue and rhodamine-dextran in red. Scale bars, 100 μ m. Representative images captured 20 min after rhodamine-dextran administrations from independent experiments ($n = 2$ mice/group) are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. indicates not significant ($P > 0.05$). Statistics calculated using unpaired t test.

Figure 4.

Differential effects of CD45-SAP and irradiation on blood and thymus. **(a)** Relative levels of peripheral myeloid cells in (non-transplanted) C57BL/6 mice at various times post 3 mg/kg CD45-SAP or 5Gy TBI. Data represent mean percentage relative to non-treated control mice $\pm$ s.e.m. ($n = 20$ mice/group, $n = 4$ mice per time point, assayed individually). **(b)** Kaplan-Meier survival curve following systemic *Candida albicans* infection 2 d after conditioning and in non-conditioned C57BL/6 control mice ($n = 10$ mice/group). **(c)** Relative levels of peripheral CD3+ T cells at various times after administration of 3 mg/kg CD45-SAP or 5Gy TBI in (non-transplanted) C57BL/6 mice. Data represent mean percentage relative to non-treated control mice $\pm$ s.e.m. ($n = 20$ mice/group, $n = 4$ mice per time point, assayed individually). **(d)** Hematoxylin and eosin staining of thymus (500 μ m scale bars) and thymic cortex (50 μ m scale bars) from non-treated control C57BL/6 mice and 3 mg/kg CD45-SAP or 5Gy TBI conditioned mice 2 d after conditioning. **(e)** Absolute number of T-cell receptor excision circles (TRECs) per mg of thymus tissue 3 d after conditioning with 3 mg/kg CD45-SAP or 5Gy TBI. Control represents non-treated mice. Data represent mean $\pm$ s.d. ($n = 4$ mice/group, assayed individually). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. indicates not significant ($P > 0.05$). Statistics calculated using unpaired t test.

Figure 5.

Correction of sickle cell disease. **(a)** Experimental outline for CD45-SAP conditioning and transplantation in sickle disease mice. CD45-SAP dosing (in mg/kg) schedule is shown and BM represents transplantation of 10 million wild-type bone marrow cells. **(b)** Donor myeloid chimerism 4 months after transplantation of sickle mice transplanted under the conditions in **a**. Data represent the mean $\pm$ s.d. ($n = 18$ mice, $n = 6$ mice/group, assayed individually). **(c)** Assessment of red blood cell (RBC), hemoglobin (Hgb), hematocrit (Hct), and reticulocyte (Retic) numbers in wild-type control mice, sickle disease mice and group A (corrected sickle mice) 4 months after transplantation. Data represent the mean $\pm$ s.e.m. ($n = 6$ mice/group, assayed individually). **(d)** Native-PAGE analysis of normal (Hba) and sickle (Hbs) hemoglobin protein in blood from wild-type control mice, sickle mice and group A mice (two representative mice from each group). Statistics calculated using unpaired *t* test. Blot of additional groups shown in Supplementary Figure 10f. **(e)** Representative peripheral blood smears of wild-type mice, sickle disease mice, and group A mice, with sickle cells indicated by arrows (20 μ m scale bars). **(f)** Representative spleens from wild-type control mice, sickle mice and group A mice (two representative mice from each group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s., not significant ($P > 0.05$).